Colloids and Surfaces A: Physicochem. Eng. Aspects 506 (2016) 739–746

Contents lists available at ScienceDirect

Colloids and Surfaces A: Physicochemical and Engineering Aspects

journal homepage: www.elsevier.com/locate/colsurfa

Stability of polymersomes prepared by size exclusion chromatography and extrusion

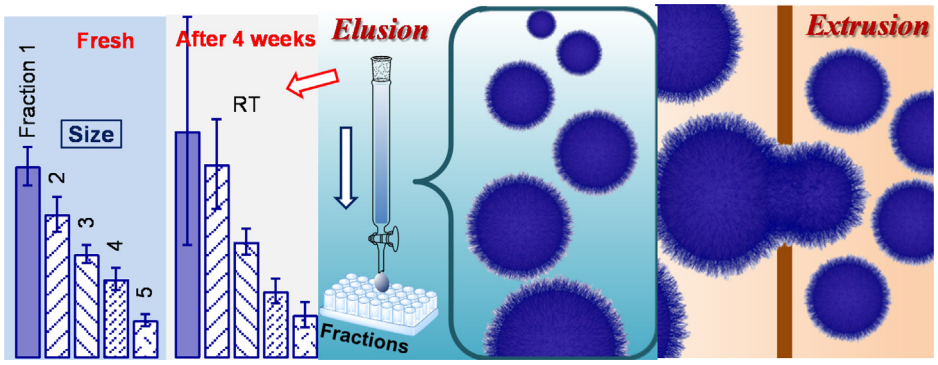
Julia E. Bartenstein[a], James Robertson[b], Giuseppe Battaglia[c], Wuge H. Briscoe[a,*]

[a] School of Chemistry, University of Bristol, Cantock's Close, Bristol, BS8 1TS, UK
[b] Department of Biomedical Science, University of Sheffield, Sheffield, UK
[c] Department of Chemistry, Gordon Street, University College London, WC1H 0AJ, London, UK

HIGHLIGHTS

- Polymersomes from film rehydration are polydisperse.
- A narrow size distribution is obtained from extrusion and size exclusion chromatography.
- Polymersome size from SEC increases over time due to elusion dilution.
- Extruded polymersomes are more stable.
- Storage temperature, buffer, and dilution all affect polymersome stability.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 21 April 2016
Received in revised form 11 July 2016
Accepted 15 July 2016
Available online 25 July 2016

Keywords:
Polymersomes
Polymer vesicles
Size exclusion chromatography

ABSTRACT

In this work, stability of poly(butadiene)-poly(ethylene oxide) (PBD-PEO) polymersomes, self-assembled from two polymers with different molecular weights (PBD_{32}-PEO_{21} and PBD_{125}-PEO_{80}) in either pure H_2O or phosphate buffered saline (PBS), is studied. Polymersome dispersions usually show large polydispersity, and it is thus desirable to separate different-sized vesicles if a narrow size distribution is required, *e.g.* for model systems in certain applications. This is typically achieved by extrusion through a membrane with a designated pore size or, less commonly, by size exclusion chromatography (SEC). Here, we find that both extrusion and SEC of polymersome dispersions with vesicle sizes ranging from 100 to 5000 nm and polydispersity index (PDI) = 1, can yield smaller vesicles with PDIs < 0.35. With SEC, it is possible to separate fractions of polymersomes with different sizes. However, the SEC polymersome size and particularly the spread in the size increase significantly over time, whereas the extruded polymersomes are shown to be more stable. We attribute this to possible dilution of the polymersome dispersion during the SEC elusion process. The effects of temperature and the PBD-PEO molecular weight on the stability of the extruded polymersomes against dilution in pure water and phosphate buffer are further studied. It is found that the polymersomes show higher stability when stored at lower temperature, undiluted, and prepared in phosphate buffer, whereas the polymer molecular weight does not have a large influence on the stability.

1. Introduction

Polymersomes are hollow polymer spheres typically of size ~100 nm – a few μm, self-assembled from amphiphilic

* Corresponding author.
E-mail address: wuge.briscoe@bristol.ac.uk (W.H. Briscoe).

http://dx.doi.org/10.1016/j.colsurfa.2016.07.032

block-copolymers of appropriate molecular architecture. They have been widely studied and their characteristics and applications have been the focus of a number of review articles [1–5]. Preparation of polymersomes is usually achieved by film rehydration (also called thin film hydration) [6–8], electroformation [9–11] or solvent switch methods [12–14], although other processes such as the pH switch [15,16] or inverted emulsion techniques [17,18] have also been reported. The process of vesicle formation from film rehydration has been followed *in situ* using optical microscopy, which showed that aggregates evolved from dot-like particles to smaller vesicles in 30 min, which eventually fused into larger polymersomes [19]. During their formation, polymersomes initially adhered to the sides of the reaction vial in stacks, and the fully assembled vesicles could subsequently be dispersed in solution by stirring [19].

It has been reported that, under certain preparation conditions and using certain copolymers, vesicles with a narrow size distribution could be formed, *e.g.* poly(ethylene oxide)-*b*-poly(*N,N*-diethylaminoethyl methacrylate) (PEO-*b*-PDEAMA) polymersomes (diameter–90 nm) prepared by the pH switch method [15]. However, commonly employed preparation procedures give rise to vesicle dispersions with high polydispersity indices (PDIs) [20–22]. Hence for applications where a narrow size distribution is desirable, it is required to separate different aggregate sizes. The most common approach is extruding the dispersion through a polycarbonate membrane with a certain pore size. During extrusion, polymer aggregates with diameters smaller than the pore size can pass the membrane, whereas larger aggregates either deform or rupture, undergoing reassembly. For instance, ~150 nm poly(dimethylsiloxane)-poly(2-methyloxazoline) (PDMS-PMOXA; MW = 7300 g mol^{-1}) polymersomes were obtained after the initial polydisperse polymersome dispersion was extruded through a 200 nm membrane [23]. Similarly, extrusion of poly(ethylene oxide)-poly(propylene oxide)-poly-(ethylene oxide) (PEO-PPO-PEO; MW = 4400 g mol^{-1}) vesicles through a 100 nm membrane gave vesicle sizes of ~50–100 nm [24]. Also, extrusion of poly(butadiene)-poly(ethylene oxide) (PBD-PEO; MW = 3500 g mol^{-1}) polymersomes, with initial sizes of up to 50 μm, through a 100 nm membrane yielded monodisperse dispersions with vesicle sizes < 100 nm [25]. In a different study, extrusion of PBD-PEO polymersomes made from low molecular weight copolymers (MW = 3600 g mol^{-1}) through a 100 nm membrane gave vesicles of size ~120 nm [26]. PBD-PEO vesicles prepared from high molecular weight polymers (MW = 10,400 g mol^{-1}) were extruded through a 250 nm membrane first, before being passed through a 100 nm membrane, which gave vesicle sizes comparable to the lower molecular weight sample [26].

Another approach to separate polymersome sizes is *via* size exclusion chromatography (SEC), although this method has not been widely reported. The SEC method is based on the principle that aggregates of different sizes will pass through a column filled with small polymer beads at different speeds and therefore will be eluded at different times. Hence, this method allows for collection of a range of polymersome sizes from an initial dispersion. This method has been utilised, *e.g.* for purification of ~100 nm fluorescently poly(2-(methacryloyloxy)ethyl phosphoryl-choline)-poly(2-(diiso-propylamino)ethyl methacrylate (PMPC-PDPA) and PEO-PDPA, *i.e.* separating polymersomes from free polymers and dyes [27]. However, the SEC method was not specifically utilised to separate different polymersome sizes, and no discussion or investigation of the obtained polymersomes was pursued in that study. We note that a study has just appeared (during the revision of this manuscript) to use SEC to separate different sizes of polymersomes, and again the stability of the obtained polymersomes was not investigated [28].

The long-term stability of the polymersome dispersions has been investigated in a number of studies [29–31] and is frequently compared to the stability of liposomes. Although polymersomes bear a structural resemblance with liposomes, they are considered more stable, because of their thicker membranes facilitated by higher molecular weight of the hydrophobic polymer segments compared to thinner lipid bilayers enclosing a liposome. Furthermore, the versatility of polymer synthesis allows the potential for a wide range of chemical functionalities to be incorporated into polymersomes [32,33]. To enhance liposome stability, poly(ethylene glycol) (PEG) polymers, and less commonly other polymers [34], are often adsorbed on the vesicle surface to provide additional steric repulsions [35]. However, PEG stabilised liposomes are still less stable than PEG containing polymersomes of similar sizes (~100 nm) [26], again pointing to the superior stability of polymersomes over liposomes.

The stability of polymersomes is influenced by a number of factors. For example, polymersomes tend to show a higher stability when self-assembled from a block copolymer with a higher molecular weight hydrophobic block, as the thickness of the membrane increases with increasing hydrophobic chain length [38]. Furthermore, polymer vesicles show high stabilities when stored at a low temperature [31]. For instance, Li et al. [36] showed that PBD_{219}-PEO_{121} polymersomes prepared *via* film rehydration, loaded with paclitaxel and extruded through a 200 nm membrane, were stable for 4 months at 4 °C. Additionally, as polymersomes in bulk solution are in thermodynamic equilibrium, their stability is also affected by solution conditions, *e.g.* solvency and polymer/polymersome concentration. Luo and Eisenberg [37], for example, showed that changing the water content (between ~25% and ~67%) of a water/THF/dioxane solvent mixture could result in fusion and fission of poly(styrene)-*b*-poly(acrylic acid) (PS-*b*-PAA) polymersomes. The sizes of the vesicles could be altered reversibly from ~90 nm (for 25% water) to ~200 nm (for 67% water). In another study, an increase in stability was found for liposomes prepared in phosphate buffered saline (PBS) instead of pure water [38], although such an effect has not been reported for polymersome stability.

In this work, we have studied the stability of PEO-PBD polymersome dispersions prepared by the film rehydration technique. Subsequently, the polydisperse polymersomes were either extruded or passed through a SEC column in order to obtain dispersions with a narrow size distribution. The stability of the resultant polymersome dispersions was compared. The SEC method has a potential advantage of extracting polymersomes of a number of different sizes, although it has not been widely studied previously. We thus further compared the stability of the polymersome dispersions from the SEC and extrusion method prepared in either PBS or water from two different PEO-PBD molecular weights against temperature and dilution. It was found that the vesicles prepared by the SEC method were not stable over prolonged time, which was attributed to a change in the thermodynamic conditions caused by dilution of the vesicle dispersions as they were eluded through the column. Extruded samples showed better stability, especially when stored at lower temperatures and prepared in a PBS solution instead of pure water. However, the PBD-PEO molecular weight did not show a significant impact on the polymersome stability.

2. Experimental methods and materials

2.1. Chemicals and methods

All chemicals were acquired commercially and used as received unless otherwise stated. PBD-PEO diblock copolymers (Fig. 1) were purchased from Polymer Source (Canada). Phosphate buffered

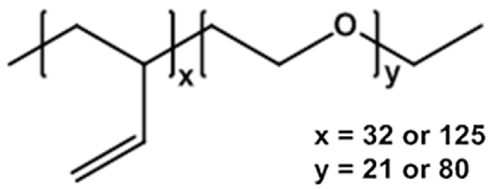


Fig. 1. Chemical structure of PBD_{32}-PEO_{21} and PBD_{125}-PEO_{80} used for preparation of polymersomes.

saline (PBS) was obtained from Sigma Aldrich (Dulbecco's PBS, Sigma Aldrich, UK). MilliQ water with a resistivity of 18.2 MΩ cm and a total organic content (ToC) ~3 ppb was used throughout. All samples were stored under ambient conditions unless otherwise indicated. Dynamic light scattering (DLS) characterization was conducted using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK) at 25 °C. The polydispersity index (PDI) referred to in this manuscript is a dimensionless quantity, indicating the broadness of size distribution determined by the Cumulants analysis from DLS measurement. A perfectly monodisperse sample would have a PDI < 0.05, whereas a PDI greater than ~0.7 indicates that the sample has a very broad size distribution. Copper grids for TEM were carbon coated and a droplet of the sample was applied to the carbon grid and incubated for 1 min before excess polymersome dispersion was blotted away. The sample was imaged using a JEOL 1400 TEM (USA).

2.2. Preparation of polymersomes

PBD-PEO polymersomes were prepared by film rehydration [39]. Briefly, 0.05 g of diblock copolymer was dissolved in 5 mL of $CHCl_3$ in a round bottom flask. The solvent was then evaporated under reduced pressure to give a thin polymer film on the wall of the vial. Then 4.95 mL of either MilliQ water or PBS was added and the resulting dispersion was stirred at room temperature (RT) overnight before being sonicated for 20 min. The DLS measurement showed that the obtained cloudy dispersion contained polymer aggregates (polymersomes, micelles and larger aggregates) of different sizes in the range of ~100–5000 nm, and this polydisperse sample was then used for either extrusion or size exclusion column chromatography (SEC).

Extrusion was performed using a mini lipid extruder (Avanti® Mini-Extruder, Avanti Polar Lipids, USA) with either 100 nm or 400 nm polycarbonate membranes (Avanti Polar Lipids, USA). During extrusion, small vesicles can pass through the membrane, whereas larger ones are deformed or rupture and then re-assemble. However, small polymer assemblies, such as micelles, can pass through the membrane, making the presence of such nanoparticles inevitable in the extruded dispersion. Extrusion of the polymer aggregates from film hydration above through a 400 nm membrane resulted in low polydispersity indices (PDIs); however, extrusion through a 100 nm membrane gave higher PDIs (~0.5). Hence, to optimise size separation, all samples were extruded first through a 400 nm membrane, to break up bigger aggregates, before being passed through the 100 nm membrane. With this method, small PDIs (<0.2) were achieved.

Alternatively, polymersomes of different sizes were separated using an SEC column. Before a sample was passed through a size exclusion column, it was concentrated using a MikroKros filter module with 11 cm^2 surface area (Spectrum Labs, USA) until a volume of ~0.3 mL was obtained. Then a size exclusion column (Chromaflex, Kontes, USA) was filled with Sepharose (Sepharose 4B, bead diameter ~45–165 μm, average pore size ~30 nm for an average 90 μm bead, Sigma Aldrich, UK) and washed with either MilliQ water or PBS. Sepharose is a crosslinked form of agarose and chemically inert, and Sepharose beads are commonly used for the analysis of polymers (*e.g.* to separate different polymer molecular weights in a polydisperse polymer sample). The sample was applied on top of the column, eluded with water or PBS and the resulting fractions were collected in cell culture plates and then analysed by DLS. The elusion process was completed typically in 15 min. The elusion time was ~5 s per fraction (2 drops) and after initial DLS, fractions which had polymersomes of same size were combined and DLS was re-measured (PDIs < 0.4).

3. Results and discussion

3.1. Size exclusion chromatography (SEC) vs. extrusion

Poly(butadiene)-poly(ethylene oxide) (PBD-PEO) polymersome dispersions in H_2O or phosphate buffered saline (PBS) prepared from film rehydration showed large polydispersity (PDI–1) with sizes ranging from ~100 to 5000 nm. These polydisperse polymersome dispersions were used in further experiments to obtain polymersomes with a narrower size distribution. Such size separation is most commonly achieved by sonication and extrusion [39], however, size exclusion chromatography (SEC) can also be used, albeit it has not been commonly used for polymersomes. We have compared the stability of the polymersomes obtained using these two methods.

Using SEC, a range of vesicle sizes could be obtained for PBD_{32}-PEO_{21} and PBD_{125}-PEO_{80} polymersomes in MilliQ and PBS from the same film hydration batch. The size separation is achieved by passing the dispersion through the column filled with porous Sepharose beads. Because of the small pore sizes of the beads, only smaller aggregates can penetrate the pores whereas bigger vesicles will not interact with the beads strongly and will hence be eluded through the column faster.

Typical vesicle sizes obtained for PBD_{32}-PEO_{21} and PBD_{125}-PEO_{80} polymersome samples in H_2O and PBS from an SEC procedure are listed in Table 1. Example DLS data for PBD_{32}-

Table 1
Vesicle diameters d and PDIs for polymersomes in H_2O and PBS using the SEC method for two different PBD-PEO molecular weights. The PDI values are given in the brackets after the polymersome diameter values.

Fraction	Polymersome size *d* (nm) and (PDI)			
	PBD_{32}-PEO_{21} in H_2O	PBD_{32}-PEO_{21} in PBS	PBD_{125}-PEO_{80} in H_2O	PBD_{125}-PEO_{80} in PBS
1	566 (0.20)	1226 (0.28)	460 (0.27)	387 (0.32)
2	385 (0.28)	737 (0.34)	287 (0.26)	341 (0.28)
3	347 (0.29)	487 (0.25)	147 (0.31)	116 (0.26)
4	252 (0.35)	448 (0.27)	68 (0.56)	86 (0.45)
5	220 (0.31)	379 (0.33)		55 (0.77)
6	190 (0.32)	307 (0.23)		
7	116 (0.31)	179 (0.37)		
8	(15, micelles)	97 (0.34)		

Table 2
PBD-PEO polymersome sizes extruded through 100 nm and 400 nm membranes in H_2O or PBS. The PDI values are given in the brackets after the polymersome diameter values.

Polymersomes	Polymersome size *d* (nm) and (PDI)			
	100 nm extrusion membrane		400 nm extrusion membrane	
	in H_2O	in PBS	in H_2O	in PBS
PBD_{32}-PEO_{21}	169 (0.15)	156 (0.11)	364 (0.09)	470 (0.11)
PBD_{125}-PEO_{80}	202 (0.10)	194 (0.13)	362 (0.10)	410 (0.15)

PEO_{21} polymersomes in H_2O before and after SEC size separation is shown in Fig. S1 and S2 respectively in the ESI section. The table shows that, for example for a PBD_{32}-PEO_{21} polymersome sample in H_2O, 7 different vesicle fractions with sizes ranging from ~570 nm to 115 nm were collected (PDIs < 0.35), in addition to a micellar dispersion. Samples prepared in PBS and/or made from higher molecular weight polymers showed similar behaviour, with polymersome sizes ranging from 100 to 1200 nm obtained at different elusion times. Smaller, and a smaller size range of, polymersomes were obtained with the higher molecular weight polymer (PBD_{125}-PEO_{80}). Sizes smaller than 100 nm usually showed higher PDIs, which might be due to the presence of micelles. As the polymersome size gets closer to the micelle size (~15 nm), the aggregates cannot easily be separated and hence fractions with small sizes are most likely mixed micelle-vesicle dispersions. In general, this method allowed for separation of a range of sizes with relatively good PDIs (usually between 0.15–0.37). However, it was found that the sizes acquired differed for each film rehydration polymersome batch. The exact sizes obtained with the SEC method were thus not reproducible, varying for each individual sample. This is an interesting result, as the polymersomes do not undergo forced reassembly (as in extrusion). The DLS showed broad size distributions in the film rehydration sample. After SEC, the polymersome size distribution is no longer continuous; rather it entails a collection of discrete sizes, each possibly corresponding to local minimum in the energy landscape of polymersome self-assembly. This implies that certain degree of reassembly would have occurred during elusion, possibly due to dilution by the solvent. The fact that different film rehydration samples showed different size fractions indicates that the polymersomes obtained are not under equilibrium and remain in kinetically trapped states. This is pertinent later when we evaluate the stability of the SEC polymersomes.

In comparison, Table 2 shows the polymersome sizes obtained for PBD_{32}-PEO_{21} and PBD_{125}-PEO_{80} vesicles in H_2O and PBS, all with a fairly narrow size distribution (PDIs < 0.2), prepared *via* extrusion of the polydisperse film hydration samples. For the 100 nm membranes, the obtained polymersome sizes were always larger than the pore size of the polycarbonate membrane, indicating that the polymersomes were deformable and the limiting factor for the size was the curvature related elastic bending energy F_H in the polymersome reassembly upon extrusion which is given by Helfrich as [40]

$$F_H = \left(\frac{\kappa}{2}\right)\oint dA(2H + C_0)^2 + \kappa_G \oint dAK, \quad (1)$$

where κ is the bending rigidity, H the mean curvature, C_0 the spontaneous curvature, κ_G the elastic modulus of the Gaussian curvature, A the area of the membrane and K a topological invariant.

PBD_{125}-PEO_{80} polymersomes showed larger sizes than PBD_{32}-PEO_{21} vesicles, suggesting that the higher molecular weight polymersomes were more elastically robust and could be deformed more than the lower molecular weight sample without rupturing. Thicker membranes (~15 nm for PBD_{125}-PEO_{80} *vs.* ~9 nm for PBD_{32}-PEO_{21})[37] display higher bending rigidities (*i.e.* higher κ and κ_G in Eq. (1) and hence larger F_H, which means that the PBD_{125}-PEO_{80} polymersomes would adopt a smaller curvature C due to higher bending energy cost, as the energetic balance is examined while they are squeezed through the membrane pores.

Extrusion through the 400 nm membranes, on the other side, gave rise to polymersome sizes ranging from 360 to 470 nm, comparable to the membrane pore size. Here, larger sizes were obtained for dispersions in PBS for both polymer MWs, suggesting that the steric repulsion between the PEO segments was somewhat reduced in the presence of PBS. As a result and in accordance with the packing parameter [41], a denser packing of the hydrophilic PEO segments and an associated smaller effective area would lead to a smaller curvature of the polymersomes. A similar effect has been observed for addition of salt to micelles [42]. Polymersomes from two different PEO-PBD MWs, however, showed similar vesicle sizes *d*, suggesting that the polymer size was limited by the membrane pore size which would outweigh the difference in the elastic bending energy F_H associated with the polymersome membrane thickness which depends on the MW of the hydrophobic PBD segment (and that of the overall polymer).

However, as for the SEC samples, variations in the polymersome size (up to 100 nm) from different batches of film rehydration samples were observed. This suggests that, similar to the SEC method above, the extruded vesicles probably did not reach equilibrium and their size would not have been optimised for the thermodynamic consideration of the bending energy; rather it was templated by the membrane pore size, and the polymersomes existed in a kinetically (temporarily) stable state. This is borne out as the stability of the polymersomes against time from both the SEC and extrusion methods is compared below.

Table 3
Sizes collected for PBD_{32}-PEO_{21} and PBD_{125}-PEO_{80} polymersomes in H_2O and PBS using SEC. DLS was performed for fresh dispersions and after storage for 4 weeks. The PDI values are given in the brackets after the polymersome diameter values.

Fraction	Polymersome size *d* (nm) and (PDI)								
	PBD_{32}-PEO_{21}					PBD_{125}-PEO_{80}			
	in H_2O			in PBS		in H_2O		in PBS	
	fresh	4 weeks	4 °C	fresh	4 weeks	fresh	4 weeks	fresh	4 weeks
1	1143 (0.20)	1353 (~1)	864 (0.54)	1226 (0.28)	1532 (0.83)	460 (0.27)	2461 (~1)	387 (0.32)	465 (0.52)
2	857 (0.24)	1157 (0.46)	974 (0.33)	737 (0.34)	898 (0.47)	287 (0.26)	473 (0.48)	341 (0.28)	1945 (~1)
3	621 (0.17)	695 (0.22)	905 (0.38)	487 (0.25)	675 (~1)	147 (0.31)	235 (0.66)	116 (0.26)	279 (~1)
4	473 (0.28)	401 (0.37)	395 (0.65)	448 (0.27)	713 (~1)	68 (0.56)	129 (~1)	86 (0.45)	216 (~1)
5	227 (0.31)	260 (0.58)	223 (0.47)	379 (0.33)	463 (0.64)			55 (0.77)	70 (~1)
6				307 (0.23)	551 (~1)				
7				179 (0.37)	277 (~1)				
8				97 (0.34)	182 (~1)				

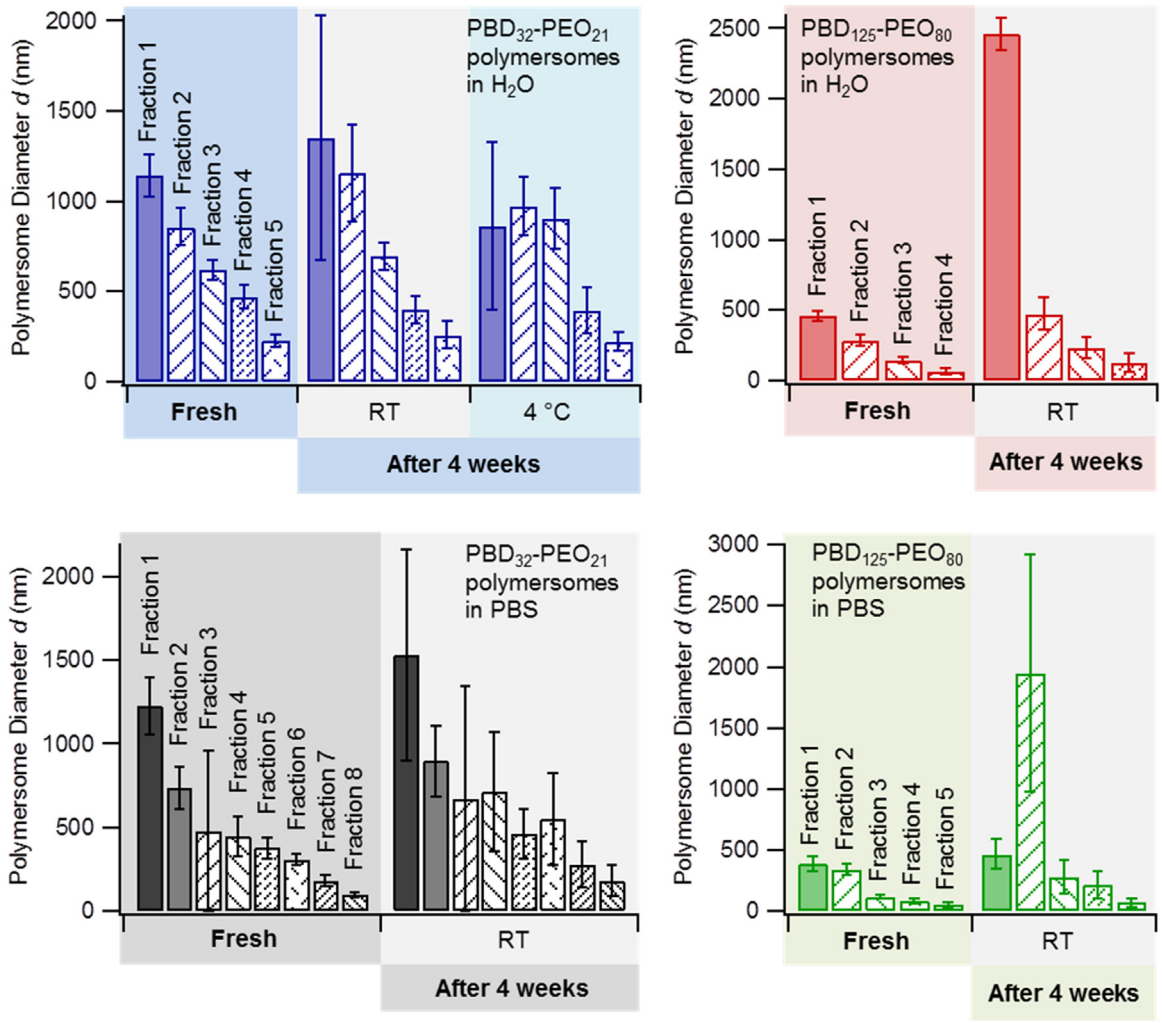


Fig. 2. Polymersome diameters (d) of different SEC fractions of PBD_{32}-PEO_{21} and PBD_{125}-PEO_{80} polymersomes in H_2O and PBS. The DLS d values were recorded from fresh polymersome dispersions and after storage for 4 weeks at room temperature (RT) and also at 4 °C (in the case of PBD_{32}-PEO_{21} H_2O dispersions). The broadness of the polymersome size distribution as indicated by the error bars, calculated from the DLS PDI values, was uniformly larger after 4 weeks of storage for each fraction.

3.2. Stability of polymersome dispersions

3.2.1. Stability of polymersomes size separated by size exclusion chromatography (SEC)

Vesicle sizes in freshly prepared SEC dispersions ranged from ~100–1200 nm (Table 1). To study their stability against time, the samples in both H_2O and PBS were stored at room temperature for 4 weeks, and then analysed by DLS. PBD_{32}-PEO_{21} polymersomes in H_2O were further studied after storage in the fridge (4 °C) for 4 weeks.

For example, five fractions of the PBD_{32}-PEO_{21} SEC polymersomes in water were obtained and their sizes (d) are listed in Table 3 and also shown in Fig. 2. Also shown are the sizes after storage at RT for 4 weeks. The PDIs in the polymersome size after 4 weeks were increasing for all fractions. The polymersome size increased significantly for most fractions, with the biggest size increase observed for the 2nd fraction (initially 857 nm), which showed a size of 1157 nm after 4 weeks RT storage. In contrast, it was also observed that sizes decreased during 4 weeks (4th fraction), however, a decrease in polymersome size was the exception. This confirms that the SEC polymersomes were not in the equilibrium sizes, and the bending energy F_H (Eq. (1)) was reduced as the curvature C of the polymersomes was reduced. Such a size increase was observed for all experiments conducted, for samples prepared from both lower and higher molecular weight and dispersions in PBS and water. No clear trend was observable and sizes varied greatly with each sample, and with different experiments. The PDIs of the samples also increased greatly, with some samples having PDIs ~1 after 4 weeks.

PBD_{32}-PEO_{21} SEC polymersomes in water were further studied after storage at 4 °C for 4 weeks (Table 3 and Fig. 2). It was assumed that storage at lower temperature would slow down coalescence and the increase in the polymersome size; however, samples stored at lower temperature did not always follow this trend. For example, the 1st fraction decreased in size from 1143 nm to 864 nm. The same fraction increased in size when stored at RT (to 1353 nm), which indicates that the temperature influences F_H. In addition, the 3rd fraction (621 nm) did show a bigger size increase when stored at 4 °C (905 nm) than when stored at RT (695 nm), which is counterintuitive to an expected slowing down of size increase.

The size change of the SEC vesicles might be explained by the fact that the polymersome dispersions were diluted after the eluding process. The dilution would cause a change in the concentration of the polymers in the bulk undergoing dynamic self-assembly, and in turn the energetic balance considerations in the polymersome assembly, and such a dilution effect could vary from fraction to fraction. As a result, the polymersomes would undergo reassembly over time, leading to the observed larger sizes. Similar behaviours have also been observed by Luo and Eisenberg, who studied size

Table 4
PBD-PEO polymersome sizes extruded through 400 nm membranes for freshly prepared dispersions and after 4 weeks of storage at RT and 4 °C. The PDI is given in brackets after the polymersome diameter.

Polymersome size d(nm) and (PDI) in H_2O					
Polymersomes	Fresh	After 4 weeks			
		RT		4 °C	
	Dilution →	1×	10×	1×	10×
PBD_{32}-PEO_{21}	485 (0.09)	416 (0.15)	1130 (~1)	487 (0.17)	422 (0.19)
PBD_{125}-PEO_{80}	384 (0.11)	350 (0.13)	1140 (~1)	350 (0.18)	330 (0.36)
Polymersome size d(nm) and (PDI) in PBS					
Polymersomes	Fresh	After 4 weeks			
		RT		4 °C	
	Dilution →	1×	10×	1×	10×
PBD_{32}-PEO_{21}	374 (0.10)	388 (0.15)	395 (0.21)	364 (0.15)	352 (0.26)
PBD_{125}-PEO_{80}	422 (0.14)	383 (0.18)	417 (0.23)	436 (0.15)	431 (0.22)

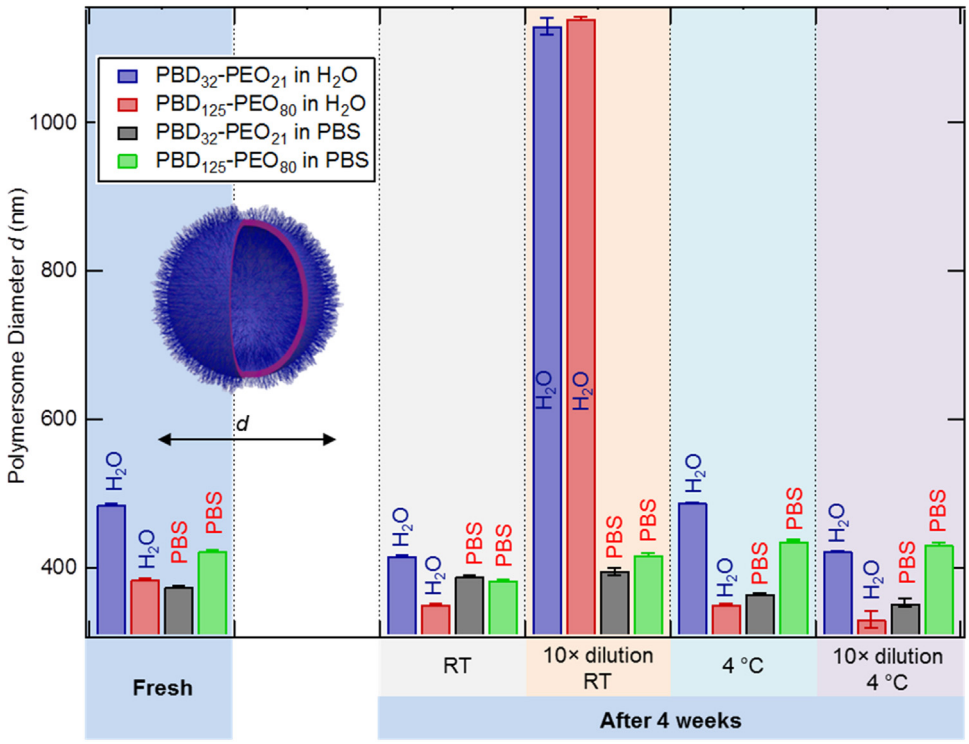


Fig. 3. Extruded polymersomes were stored under different conditions (at RT or at 4 °C, undiluted or after 10 × dilution with either H_2O or PBS) and the sizes of the freshly prepared dispersions were compared to the sizes of the vesicles after 4 weeks storage.

control of poly(styrene)-*b*-poly(acrylic acid) (PS-*b*-PAA) vesicles [37]. When the water content in a water/THF/dioxane solution of polymersomes was increased, an increase in the vesicle size was observed. This was explained by an increase in interfacial energy with increasing water content, and hence the polymersomes minimized the interfacial area by increasing in size, which minimised the overall interfacial area. Mechanistically, such an increase in size could be achieved by either swelling or fusion of polymersomes, although fusion was suggested to be the cause in this particular study [37]. By comparison, this dilution effect is much less prominent in the extrusion process, leading to better polymersome size stability over time, as we describe below.

3.2.2. Stability of extruded polymersomes

Four samples (PBD_{32}-PEO_{21} and PBD_{125}-PEO_{80} extruded through a 400 nm membrane in either MilliQ or PBS) were analysed by DLS and then divided into four aliquots, each of which was stored under a different condition. The 1st batch was stored at room temperature (RT), the 2nd was diluted by a factor of 10 before being stored at RT, the 3rd was stored in a fridge (4 °C), and the 4th was stored in the fridge after being diluted by a factor of 10. After four weeks, the sample sizes were analysed by DLS as listed in Table 4 and also illustrated in Fig. 3.

The size of the both MW PBD-PEO polymersomes in water did not show significant increases or changes after 4 weeks when stored undiluted or when stored at 4 °C. An increase in the PDI values however suggests that some relaxation in the vesicle size has occurred, leading to a slightly broader size distribution. However, diluted samples stored at RT more than doubled in size over a period of 4 weeks and also showed large PDI (~1). This is consistent with the SEC samples discussed above where we attributed the vesicle size increase to dilution during the eluding process, which would perturb the thermodynamic solution conditions for self-assembly. Mechanistically, such a size increase can be caused by swelling or fusion of polymersomes. The size increase due to dilution appeared to be slowed down at the lower temperature and samples stored

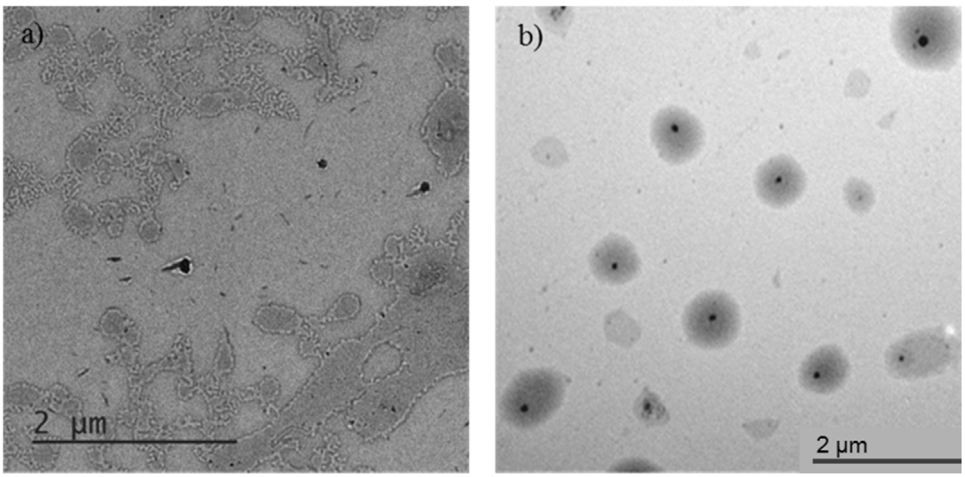


Fig. 4. TEM images taken after 3 months of storing an undiluted sample at room temperature for PBD_{125}-PEO_{80} polymersomes in a) H_2O and b) PBS.

in the fridge were stable over the test period, with a slightly larger PDI value.

One would expect that polymersomes made from higher MW polymers would be more stable, as the membrane thickness, and hence the robustness of these assemblies, is enhanced by the molecular weight [43], but both 10 × diluted PBD_{32}-PEO_{21} and PBD_{125}-PEO_{80} polymersomes showed similar size increases when stored at RT. A temperature dependent size increase was also observed by Lee et al. [31], who studied poly(ethylene glycol)-poly(D,L-lac-tide) (PEG-PLA) polymersomes stored at 4 and 35 °C. In this study, polymersomes prepared from lower molecular weight polymers increased in size over 4 days at both temperatures. The size increase, which was suggested to be due to fusion of vesicles, was higher for samples stored at 37 °C [31]. Additionally, polymersomes with longer hydrophilic block lengths were more stable, which was attributed to the steric stabilization effect. In contrast, this effect however was not observed for the PBD-PEO vesicles made from higher molecular weight hydrophilic groups examined here.

An undiluted sample of PBD_{125}-PEO_{80} polymersomes in H_2O stored at RT, the DLS size of which appeared stable over the 4 weeks period, was further analysed by TEM 3 months (stored at RT) after the vesicles were originally prepared. The TEM image in Fig. 4a shows that interconnected network of polymer assemblies had formed presumably from fusion of vesicles, indicating that polymersomes prepared in aqueous environment lacked long term stabilities.

In comparison, PBD-PEO polymersomes showed greater stabilities in PBS. As already shown in Table 4 and Fig. 3 above, PBD_{32}-PEO_{21} polymersomes in PBS showed constant sizes (size variation < 10 %) under all the conditions investigated. This indicated a stabilising effect of PBS on polymersomes, which was assumed to be due to electrostatic reasons. The stabilising effect of PBS on PEO has been suggested to be related to phosphate-PEO interactions [38]. More generally, the interactions between different ions (both cationic and anionic) with PEO have been found to depend on the valency and hydration properties of the ions [44]. This also suggests that the size increase of polymersomes is mainly due to fusion and not swelling, as the phosphate-PEO interactions deter PEO-PEO interactions. On the other hand, it is not clear how the presence of PBS would have a large impact on diffusion of solvents into the polymersome inner core in a swelling process.

PBD_{125}-PEO_{80} polymer vesicles in PBS showed similar stabilities to PBD_{32}-PEO_{21} (size variation < 15 %), but as for the H_2O samples, the higher polymer MW did not lead to higher stabilities. An undiluted sample of PBD_{125}-PEO_{80} polymersome PBS dispersions was also analysed by TEM after 3 month of storage at RT. As shown in the TEM image in Fig. 4b, the vesicles increased in size from ~420 nm (*cf.* Table 4) over that period, with the vesicle sizes ranging from 570 to 1100 nm. However, no interconnected polymer assembly networks due to vesicle fusion were observed. This further confirms that the phosphate-PEO interactions promoted polymersome stabilities, as compared to the sample prepared in water (*cf.* Fig. 4).

4. Conclusions

PBD-PEO polymersomes using two different polymer molecular weights were prepared by film-rehydration, and the polydisperse samples were further subject to either size exclusion chromatography or extrusion to produce polymersomes with a narrower size distribution. The stability of the obtained polymersome dispersions was evaluated by monitoring their size using DLS and TEM during a period up to 3 months. The effects of dilution and storage temperature were studied.

The samples prepared by size exclusion chromatography were diluted during the size separation which is believed to lead to a size increase as the polymer concentration in the bulk was reduced and the self-assembly condition was altered as a result. Hence, the polymersomes would rearrange themselves to form larger, more energetically favourable morphologies.

In comparison, extruded samples showed a greater stability and were further analysed for their stability to evaluate the effects of polymer MW, storage temperature, dilution, and PBS. The following general tendencies could be observed during the experiment: i) samples stored at lower temperature were more stable than samples stored at room temperature; ii) samples in water showed a smaller stability over time than polymersome dispersion in PBS, which could be attributed to the phosphate-PEO interactions which hindered polymersome fusion; iii) dilution tended to cause polymersome size to become less stable and encourage vesicle fusion.

Size exclusion chromatography has not been widely used to separate different polymersome sizes. We find that it is possible to obtain different vesicle sizes with a relative narrow size distribution from the same batch of film rehydration polymersome dispersions. However, the dilution effect of the SEC during the eluding process could lead to size increase over time. Our results are useful to polymersome research and applications where the vesicle stability and size distribution are important considerations.

Acknowledgements

We would like to acknowledge funding from the Engineering and Physical Science Research Council (EPSRC), the Royal Society, Taiho Kogyo Tribology Research Foundation (TTRF), the European Cooperation in Science and Technology (CMST COST) Action CM1101, and the Marie Curie Initial Training Network (MC-ITN) NanoS3 (Grant No. 290251).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfa.2016.07.032.

References

[1] J.A. Opsteen, J.J.L.M. Cornelissen, J.C.M. van Hest, Pure Appl. Chem. 76 (2004) 1309.
[2] D.E. Discher, A. Eisenberg, Science 297 (2002) 967–973.
[3] D.E. Discher, F. Ahmed, Ann. Rev. Biomed. Eng. 8 (2006) 323–341.
[4] C. LoPresti, H. Lomas, M. Massignani, T. Smart, G. Battaglia, J. Mater. Chem. 19 (2009) 3576–3590.
[5] Y. Mai, A. Eisenberg, Chem. Soc. Rev. 41 (2012) 5969–5985.
[6] M.A. Petersen, L. Yin, E. Kokkoli, M.A. Hillmyer, Polym. Chem. 1 (2010) 1281–1290.
[7] H.R. Marsden, C.B. Quer, E.Y. Sanchez, L. Gabrielli, W. Jiskoot, A. Kros, Biomacromolecules 11 (2010) 833–838.
[8] S. Rameez, U. Banerjee, J. Fontes, A. Roth, A.F. Palmer, Macromolecules 45 (2012) 2385–2389.
[9] B.M. Discher, Y.-Y. Won, D.S. Ege, J.C.-M. Lee, F.S. Bates, D.E. Discher, D.A. Hammer, Science 284 (1999) 1143–1146.
[10] M. Sauer, T. Haefele, A. Graff, C. Nardin, W. Meier, Chem. Commun. (2001), http://dx.doi.org/10.1039/B107833J,2452-2453.
[11] A. Carlsen, N. Glaser, J.-F.O. Le Meins, S.B. Lecommandoux, Langmuir 27 (2011) 4884–4890.
[12] H.R. Marsden, L. Gabrielli, A. Kros, Polym. Chem. 1 (2010) 1512–1518.
[13] J. Du, Y. Chen, Macromolecules 37 (2004) 5710–5716.
[14] H. Yang, L. Jia, C. Zhu, A. Di-Cicco, D. Levy, P.-A. Albouy, M.-H. Li, P. Keller, Macromolecules 43 (2010) 10442–10451.
[15] D.J. Adams, S. Adams, D. Atkins, M.F. Butler, S. Furzeland, J. Control. Release 128 (2008) 165–170.
[16] S.W. Kang, Y. Li, J.H. Park, D.S. Lee, Polymer 54 (2013) 102–110.
[17] E. Mabrouk, D. Cuvelier, L.-L. Pontani, B. Xu, D. Levy, P. Keller, F. Brochard-Wyart, P. Nassoy, M.-H. Li, Soft Matter 5 (2009) 1870–1878.
[18] S. Pautot, B.J. Frisken, D.A. Weitz, Langmuir 19 (2003) 2870–2879.
[19] Y. Mai, Y. Zhou, D. Yan, Small 3 (2007) 1170–1173.
[20] F. Ahmed, D.E. Discher, J. Control. Release 96 (2004) 37–53.
[21] Z. Bai, T.P. Lodge, J. Am. Chem. Soc. 132 (2010) 16265–16270.
[22] E. Cabane, V. Malinova, S. Menon, C.G. Palivan, W. Meier, Soft Matter 7 (2011) 9167–9176.
[23] K. Jaskiewicz, M. Makowski, M. Kappl, K. Landfester, A. Kroeger, Langmuir 28 (2012) 9–12636.
[24] F. Li, T. Ketelaar, M.A. Cohen Stuart, E.J.R. Sudholter, F.A.M. Leermakers, A.T.M. Marcelis, Langmuir 24 (2007) 76–82.
[25] P.P. Ghoroghchian, P.R. Frail, K. Susumu, D. Blessington, A.K. Brannan, F.S. Bates, B. Chance, D.A. Hammer, M.J. Therien, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 2922–2927.
[26] P.J. Photos, L. Bacakova, B. Discher, F.S. Bates, D.E. Discher, J. Controlled Release 90 (2003) 323–334.
[27] V. Hearnden, H. Lomas, S. MacNeil, M. Thornhill, C. Murdoch, A. Lewis, J. Madsen, A. Blanazs, S. Armes, G. Battaglia, Pharm. Res. 26 (2009) 1718–1728.
[28] J.D. Robertson, L. Rizzello, M. Avila-Olias, J. Gaitzsch, C. Contini, M.S. Magoń, S.A. Renshaw, G. Battaglia, Sci. Rep. 6 (2016) 27494.
[29] I.F. Uchegbu, Expert Opin. Drug Del. 3 (2006) 629–640.
[30] F. Li, L.H.J. de Haan, A.T.M. Marcelis, F.A.M. Leermakers, M.A. Cohen Stuart, E.J.R. Sudholter, Soft Matter 5 (2009) 4042–4046.
[31] Y. Lee, J.-B. Chang, H. Kim, T. Park, Macromol. Res. 14 (2006) 359–364.
[32] F. Meng, Z. Zhong, J. Feijen, Biomacromolecules 10 (2009) 197–209.
[33] V. Balasubramanian, O. Onaca, R. Enea, D.W. Hughes, C.G. Palivan, Expert Opin. Drug Del. 7 (2010) 63–78.
[34] R.R. Sawant, V.P. Torchilin, Soft Matter 6 (2010) 4026–4044.
[35] V.P. Torchilinl, M.I. Papisov, J. Liposome Res. 4 (1994) 725–739.
[36] S. Li, B. Byrne, J. Welsh, A.F. Palmer, Biotechnol. Progr. 23 (2007) 278–285.
[37] L. Luo, A. Eisenberg, Langmuir 17 (2001) 6804–6811.
[38] K. Kawakami, Y. Nishihara, K. Hirano, J. Phys. Chem. B 105 (2001) 2374–2385.
[39] J.C.M. Lee, H. Bermudez, B.M. Discher, M.A. Sheehan, Y.-Y. Won, F.S. Bates, D.E. Discher, Biotechnol. Bioeng. 73 (2001) 135–145.
[40] W. Helfrich, Z. Naturforsch. C 28 (1973) 693–703.
[41] J.N. Israelachvili, D.J. Mitchell, B.W. Ninham, J. Chem. Soc. Faraday Trans. 72 (1976) 1525–1568.
[42] S. Förster, N. Hermsdorf, W. Leube, H. Schnablegger, M. Regenbrecht, S. Akari, P. Lindner, C. Böttcher, J. Phys. Chem. B 103 (1999) 6657–6668.
[43] H. Bermudez, A.K. Brannan, D.A. Hammer, F.S. Bates, D.E. Discher, Macromolecules 35 (2002) 8203–8208.
[44] K.P. Ananthapadmanabhan, E.D. Goddard, Langmuir 3 (1987) 25–31.